The *Bacillus licheniformis* BlaP β-lactamase as a model protein scaffold to study the insertion of protein fragments

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Abstract

Using genetic engineering technologies, the chitin-binding domain (ChBD) of the human macrophage chitotriosidase has been inserted into the host protein BlaP, a class A β-lactamase produced by *Bacillus licheniformis*. The product of this construction behaved as a soluble chimeric protein that conserves both the capacity to bind chitin and to hydrolyze β-lactam moiety. Here we describe the biochemical and biophysical properties of this protein (BlaPChBD). This work contributes to a better understanding of the reciprocal structural and functional effects of the insertion on the host protein scaffold and the heterologous structured protein fragments. The use of BlaP as a protein carrier represents an efficient approach to the functional study of heterologous protein fragments.

Keywords: β-Lactamase; domain insertion; protein engineering; hybrid protein; chitin-binding domain; protein stability

For a long time, the structure, stability, and function of a protein have been predominantly associated with its primary structure (Tsybovsky et al. 2004). Modifications of the primary structure by point mutations have been investigated intensively and are known to alter the stability and the activity of the protein to various degrees (Hirabayashi and Kasai 1991; Lee and Levitt 1991; Palzkill and Botstein 1992; Tepper et al. 1994; Rouse et al. 1996). The effects of peptide insertions on the structure and function of proteins have, in contrast, not been investigated in detail yet (Betton et al. 1997; Collinet et al. 2000).

Peptide insertions can occur naturally as a consequence of genetic rearrangement resulting in the introduction of an amino acid sequence within an unrelated one. Such a phenomenon has been observed in large natural proteins made of at least two structural domains (Collinet et al. 2000). The result of these genetic rearrangements is the presence of discontinuous domains in natural proteins, where the linear sequence of one domain is interrupted by another inserted one. A systematic survey, indeed, indicates that 28% of structural domains are discontinuous (Jones et al. 1998). A few examples of such phenomena are found in DsbA (Martin et al. 1993), DNA polymerase

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Abbreviations: ChBD, chitin-binding domain of the human macrophage chitotriosidase; ESMS, electrospray ionization mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; DTT, dithiothreitol; DTNB, dithiobis-(2-nitrobenzoic acid); BSA, bovine serum albumin; ANS, 1-anilino-8-naphthalenesulfonate.

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(Delarue et al. 1990), and pyruvate kinase (Levine et al. 1978). These observations indicate that the sequence continuity of a structural domain is not strictly required to ensure the correct folding of the protein (Collinet et al. 2000). These events are believed to be partially responsible for the diversity of proteins. Two or more ancestral genes coding for protein domains were at some stage fused to yield proteins with new biological properties. In order to extend this evolutionary process in vitro and to design and produce recombinant proteins with new biochemical properties, it is of major importance to understand the rules that govern the conservation or alteration of the original properties of protein scaffolds upon insertion of peptides of various sizes and structures.

For this purpose, few chimeric proteins have been recently constructed. For example, Betton et al. (1997) inserted the TEM1 β-lactamase into the maltose-binding protein MalE, and the resulting chimeric protein retained the activity of both parental proteins. They showed that the bifunctionality of the hybrid protein was dependent on the insertion site, and they established that the stability of one of the chimeric protein's partners can be modulated by an independent ligand specific for the other partner of the macromolecule. Finally, they showed that the hybrid proteins were resistant to host proteases because of the decreased accessibility or flexibility of the linker peptides between the β-lactamase and MalE. Collinet et al. (2000) introduced either the dihydropholate reductase (DHFR) or β-lactamase (TEM-1) into four surface loops of phosphoglycerate kinase (PGK); these insertions caused only a small decrease of the enzymatic activity of PGK.

In order to study the structural and functional reciprocal effects of an insertion on the scaffold protein and a heterologous structured protein fragment, we have investigated the chimeric BlapChBD protein. The host protein was a natural N-terminal truncated form of the class A BlaP β-lactamase from *Bacillus licheniformis* (Frate et al. 2000). The insert was the chitin-binding domain (ChBD) of the human macrophage chitotriosidase (Tjoelker et al. 2000; Ujita et al. 2003).

The *B. licheniformis* β-lactamase BlaP is a compact protein (30 kDa) consisting of 267 residues. Its three-dimensional structure is well established (Moews et al. 1990). It comprises two domains. The α domain is only composed of helices, and the α/β domain contains both helices and β-strands. The stability and kinetic properties of this protein have been thoroughly studied, as well as its tolerance to amino acid sequence insertions (Matagne et al. 1990, 1991; Moews et al. 1990; Hallet et al. 1997; Mathonet et al. 2006).Various possible insertion sites located in loops and in secondary-structure elements have been investigated (data not shown). The most promising results have been obtained for the loop connecting α-helices 9 and 10, between residues Asp 197 and Lys 198 (Fig. 1). This loop is not directly involved in the catalytic site. This protein, expressed by *B. lichenformis*, an efficient protease producer, is naturally more resistant to proteases than its counterparts in many other prokaryotic organisms. Finally, this β-lactamase is easily overexpressed and secreted in the periplasmic space when produced in *Escherichia coli*. It therefore constitutes a unique system to use as a protein carrier.

The inserted domain, ChBD, is a small globular domain of 73 residues that binds insoluble chitin and that is naturally present in human chitotriosidase, a 50-kDa protein. This domain was chosen for conferring a binding activity to BlaP. It contains three disulfide bridges essential for its binding activity and stability (Tjoelker et al. 2000; Ujita et al. 2003). It is of interest that its three-dimensional structure has not been solved and that it is extremely difficult to produce the ChBD alone. In addition, the insertion of a domain without any preliminary knowledge of its native conformation represents an additional challenge.

The chimeric protein (BlaPCHBD) retained both the β-lactamase activity and the ability to bind to chitin. The bifunctionality of the hybrid protein allowed the relative association constant (K_r) between the chimeric protein and insoluble chitin to be determined using the β-lactamase activity as reporter. The effect of the ChBD insertion on the β-lactamase stability was also investigated by differential scanning calorimetry (DSC), far-UV circular dichroism (far-UV CD), and fluorescence spectroscopy.

To our knowledge, it is the first time that a complete 8-kDa domain whose tertiary structure is not known was successfully inserted into a host protein. This result demonstrates the efficiency of domain insertion into BlaP for the analysis of the properties of carbohydrate-binding domains. The conservation of the enzymatic activity

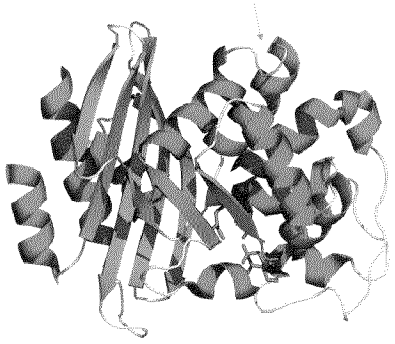


Figure 1. Ribbon diagram of the three-dimensional structure of the *Bacillus licheniformis* BlaP β-lactamase. The insertion site is indicated by an arrow. It is located in a loop connecting helix 9 to helix 10 between residues Lys 198 and Leu 199. The structure of BlaP was determined by Moews et al. (1990).

should allow the use of the β-lactamase as a reporter enzyme to investigate the interactions between polypeptides and a broad range of potential ligands.

Results

Construction of the chimeric BlaPChBD protein

The genetic construction (Fig. 2) encoding ChBD inserted into BlaP was used to transform *E. coli* JM109 to achieve the overexpression of a soluble chimeric BlaPChBD protein in the periplasmic space. The protein containing a poly-histidine tag at the C-terminal end was extracted from the periplasm by an osmotic cold shock and purified by chelation affinity and anion exchanger chromatographies (see Materials and Methods).

Constitutive expression of the parental β-lactamase BlaP in *E. coli* confers resistance to ampicillin, a system allowing a positive selection of bacteria that express chimeric proteins that retain the β-lactamase activity, such as BlaPChBD.

The integrity of the chimeric protein was verified by both N-terminal sequencing using the Edman degradation procedure and electrospray ionization mass spectrometry (ESMS). Both methods detected the presence of two forms of the protein. The most abundant form corresponds to the mature protein with the expected N-terminal TEMKD sequence. That of the second one, QATEM, corresponds to the protein with two additional residues from the signal peptide. Also, we observed a difference (183 Da) between the theoretical and experimental molecular weights of the chimeric protein. After verification of the nucleotide sequence, we performed a trypsin digestion of the protein followed by mass analysis of the resulting peptide mixture using matrix-assisted laser desorption/ionization (MALDI) (Lewis et al. 1998). Eighty-eight percent of the amino acid sequence was recovered. The uncovered region corresponded to the C-terminal extremity of the protein. From these data, we attributed the observed mass difference to a wrong procession of the poly-histidine tag in *E. coli*.

Bifunctionality of the chimeric protein

The β-lactamase moiety

To investigate the effects of ChBD insertion on the β-lactamase activity, a kinetic characterization was performed with several β-lactam substrates (Table 1). The impact of ChBD insertion differs depending on the substrate tested. For example, for nitrocefin hydrolysis, although the k_{cat} and K_m values are lowered for the chimeric protein, the k_{cat}/K_m ratio is similar to that of the host protein. For cephalothin, ChBD insertion significantly alters the k_{cat}/K_m ratio. This is essentially because of a large increase of the K_m value. In contrast, kinetic parameters for ampicillin do not seem to be modified by the insertion.

The kinetic parameters of the chimeric protein for different β-lactam substrates were also evaluated in the presence of a reducing agent (dithiothreitol, DTT). It was previously shown that mutations of any of the six cysteines within ChBD impairs binding to chitin and strongly destabilize its overall three-dimensional structure (Tjoelker et al. 2000; Ujita et al. 2003). The BlaP protein is devoid of cysteine and therefore should not be affected by the presence of DTT. The reduction of the ChBD cysteines (verified by DTNB chemical analysis) does not modify the kinetic parameters for the hydrolysis of ampicillin (Table 1). The results are different when cephalothin and nitrocefin are used as substrates. The k_{cat}/K_m ratio for nitrocefin is increased twofold in the presence of DTT; this is mostly because of a decreased K_m value. The same modifications are observed for cephalotin: a twofold increase of the k_{cat}/K_m ratio due to a decrease of the K_m value. These results demonstrate that the destabilization of the inserted domain positively modulates the activity of the host protein, probably by acting on the accessibility of the catalytic site for the β-lactam substrate.

Chitin is a crystalline insoluble substrate that prevents the determination of kinetic parameters since it interferes with absorbance measurements. For this reason, the kinetic parameters for BlaPChBD immobilized on chitin

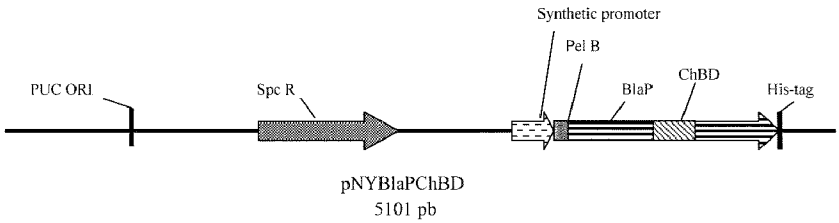


Figure 2. Plasmid pNYBlaPChBD encoding the amino acid sequence of BlaP interrupted by the ChBD gene. PUC ORI: *Escherichia coli* origin of replication from plasmid pUC; Spc R: spectinomycin resistance gene; Synthetic promoter: promoter constructed by Dr. P. Filée (Université de Liège, Belgium); Pel B: signal peptide for periplasmic secretion; BlaP: β-lactamase BlaP gene interrupted by the ChBD: gene encoding the chitin-binding domain and followed by the $(His)_7$ encoding sequence.

Table 1. *Kinetic parameters of BlaP and its chimeric derivative in the presence or absence of 5 mM DTT*

	BlaP			BlaPChBD			BlaPChBD + DTT		
Antibiotics	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (μM^{-1} sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (μM^{-1} sec^{-1})	K_M (μM)	k_{cat} (sec^{-1})	k_{cat}/K_M (μM^{-1} sec^{-1})
Nitrocefin	40 ± 2	490 ± 20	11.7 ± 0.1	20 ± 1	195 ± 5	10.3 ± 0.2	7 ± 0.1	156 ± 7	22 ± 2
Ampicillin	160 ± 1	2330 ± 10	14.8 ± 0.3	170 ± 2	2320 ± 20	13.8 ± 0.5	140 ± 4	2030 ± 10	14.3 ± 0.4
Cephalothin	25 ± 3	90 ± 10	3.6 ± 0.3	77 ± 1	75 ± 5	0.9 ± 0.3	33 ± 1	66 ± 7	2.1 ± 0.2

could not be determined. Nevertheless, we have demonstrated that the β-lactamase moiety of the chimeric protein retains its activity when immobilized on chitin powder (see below).

The ChBD moiety

To check the biological functionality of the inserted ChBD, chitin-binding assays were performed with BlaPChBD using the activity of β-lactamase as the reporter.

These experiments were performed both in the presence and absence of DTT. The oxidized ChBD retains its specific chitin-binding activity in contrast to the reduced ChBD (Table 2).

To analyze the binding properties of ChBD within the chimeric protein in more detail, we determined the relative equilibrium association constant (K_r) from the plot of binding data points as described by Gilkes et al. (1992) (Fig. 3). The calculated K_r value is 5.4 ± 0.5 L g^{-1}, a value similar to those observed for other carbohydrate-binding domains (see Discussion). In the presence of DTT, no significant interaction with the polysaccharides was reported, as observed above. This result is consistent with that reported above and underlines the requirement of the three disulfide bridges for the ChBD functionality.

At the lower protein concentrations used to determine the K_r value, BlaPChBD loses some of its enzymatic activity. This instability is significantly decreased in the presence of the polysaccharide. This is also observed for the parental β-lactamase. This could lead to underestimation of the K_r value by increasing the activity found in the supernatant after incubation of the protein with chitin when compared to protein incubated without the polysaccharide. This effect can be lessened by performing enzymatic activity measurements immediately after protein dilution. We confirmed this inactivation effect by performing kinetic experiments according to Selwyn (1965); (data not shown). This effect is significantly diminished when BSA is added.

Binding of the chimeric protein to insoluble chitin was also measured in the presence of a large concentration (100 mM) of various soluble monomers and oligomers such as *N*-acetyl-glucosamine; *N,N′*-di-N-acetylchitobiose (GlcNAc2); *N,N′,N″*-tri-N-acetylchitotriose (GlcNAc3); and *N,N′,N″,N‴*-tetra-N- acetylchitotetraose (GlcNAc4). No significant inhibition of the binding of the chimeric protein to insoluble chitin was observed, suggesting a lack of strong interactions between BlapChBD and all these soluble substrates.

Stability studies

The effects of the insertion of CHBD domain on the thermodynamic stability of BlaP were investigated using a range of techniques with urea and temperature as denaturants.

Urea denaturation

The reversibility of urea denaturation was verified by incubating both proteins overnight in 8 M urea and renaturing them by dialysis against 50 mM phosphate buffer, 150 mM NaCl (pH 7.5). No major differences were observed between the activities of the native and the refolded proteins (Table 3). Similarly, no modification of the chitin-binding properties was found after denaturant removal since the K_r values of the native and refolded state did not differ (Table 3). These results suggest that both domains are fully renatured after their treatment by urea.

Fluorescence and circular dichroism measurements also support the complete reversibility of urea denaturation as judged by the recovery of the spectroscopic

Table 2. *Binding properties of β-lactamase and the chimeric protein in the absence or presence of DTT*

Proteins	ΔA (482 nm)
BlaP	0.108 ± 0.03
BlaPChBD	1.194 ± 0.12
BlaPChBD + DTT	0.176 ± 0.04

Binding assay mixtures contained 10 mg (dry weight) of insoluble chitin and 2 μg of protein in 500 μL of 50 mM phosphate buffer, 150 mM NaCl (pH 7.5) with or without 5 mM DTT. Assay mixtures were incubated for 2 h at room temperature for binding. After removing the supernatant, the pellet was washed three times and then incubated for 2 min with 1 mL of nitrocefin (100 μM). After filtration of the suspension, absorbance of the nitrocefin hydrolysis product was measured at 482 nm.

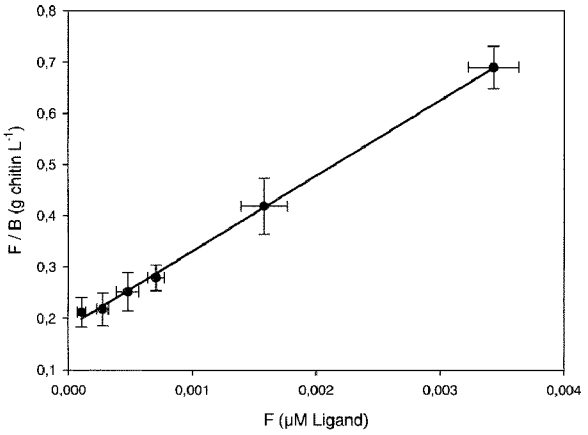


Figure 3. Binding of BlaPChBD to chitin. The binding assay mixture containing 10 mg (dry weight) of insoluble chitin and protein (25 nM to 3 µM) in 50 mM phosphate buffer, and 150 mM NaCl (pH 7.5) was incubated with rotating for 2 h at 22°C. (B) The quantity of bound ligand (in micromoles per gram of chitin); (F) the concentration of free protein (µM). Note that it was verified that the system had already reached equilibrium after 30 min of incubation.

signals after denaturant removal. Figure 4 shows the intrinsic fluorescence and far-UV CD spectra of the chimeric protein. Upon excitation at 280 nm, the maximum of fluorescence emission is 334 nm for the native and refolded proteins. Urea denaturation induces a 16-nm red shift, suggesting the loss of compact tertiary structures. The fluorescence spectra of BlaP WT and BlaPCHBD after a denaturation/renaturation cycle are virtually indistinguishable from those of the untreated native proteins. Similarly, the far-UV CD signals of the native and the refolded proteins are very similar (Fig. 5). These data confirmed that the unfolding in the presence of urea is fully reversible. The normalized unfolding transitions obtained by fluorescence and circular dichroism were similar, indicating a simple two-state denaturation mechanism for both proteins. The calculated Gibbs-free energies of unfolding and the midpoint transitions (Cm) for both proteins were derived from these curves. The small but significant difference observed between the host protein (ΔG: 38.1 ± 1.1 kJ mol^{-1}; Cm: 2.71 ± 0.03 M) and the chimeric derivative (ΔG: 34.9 ± 0.9 kJ mol^{-1}; Cm: 2.46 ± 0.06 M) suggests that the introduction of the chitin-binding domain slightly alters the stability of the overall three-dimensional structure of the hybrid β-lactamase. In addition, the ΔG value of the chimeric protein is in a range expected for a stable β-lactamase (e.g., the TEM-1 β-lactamase ΔG value is 41 kJ mol^{-1}), (Sideraki et al. 2001).

Thermal denaturation

The chimeric and parental proteins (300 nM) were incubated for 10 min at 90°C and cooled down at 25°C. The kinetic parameters using cephalothin as substrate obtained after a heating/cooling cycle (Table 3) show that thermal denaturation is fully reversible for the parental β-lactamase (97% of recovered activity). The partial renaturation of the chimeric protein after heating is characterized by a decrease of k_{cat}, whereas K_M is not altered, indicating that 43% of the protein did not refold correctly.

It has to be noticed that the complete reversibility of the parental β-lactamase thermal denaturation is only observed when the protein concentration is near 300 nM. When this concentration is increased, the refolding yield after protein heating is strongly altered.

Table 3. *Kinetic parameters of BlaP and its chimeric derivative for cephalotin*

State	Moiety	Constant (units)	Values	
			BlaP	BlaPChBD
Native	β-Lactamase	K_M (µM)	25 ± 3	77 ± 1
		k_{cat} (sec^{-1})	90 ± 10	75 ± 5
		k_{cat}/K_M (µM^{-1} sec^{-1})	3.6 ± 0.3	0.9 ± 0.3
	ChBD	K_r (L g^{-1})	/	5.4 ± 0.5
Refolded after urea treatment and dialysis	β-lactamase	K_M (µM)	25 ± 0.4	76 ± 2
		k_{cat} (sec^{-1})	106 ± 3	77 ± 5
		k_{cat}/K_M (µM^{-1} sec^{-1})	4.3 ± 0.1	1.01 ± 0.04
	ChBD	K_r (L g^{-1})	/	6.1 ± 0.7
Refolded after thermal treatment and cooling	β-lactamase	K_M (µM)	23 ± 0.5	74 ± 1
		k_{cat} (sec^{-1})	87 ± 5	43 ± 3
		k_{cat}/K_M (µM^{-1} sec^{-1})	3.8 ± 0.2	0.6 ± 0.2
	ChBD	K_r (L g^{-1})	/	2.6 ± 0.5

These parameters were determined for the native and the refolded state after chemical or thermal denaturation, allowing the evaluation of the yield of refolding after both types of denaturation.

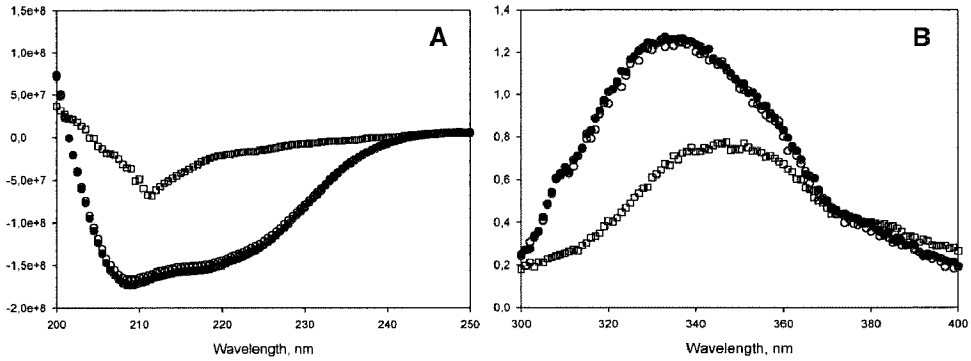


Figure 4. (*A*) Far-UV CD and (*B*) intrinsic fluorescence spectra of the chimeric protein. Spectra of the native (●), unfolded (□), and refolded (○) states were recorded in 50 mM sodium phosphate buffer (pH 7.5) in the absence or presence of 6 M urea.

A comparison of the chitin-binding activities of the unheated and heated chimeric proteins also indicated that the chitin-binding activity of the chimeric protein was altered by thermal denaturation, since only 45% of the native K_r value was recovered after one heating/cooling cycle (Table 3). By comparing the fluorescence spectra of native and refolded states (Fig. 6), it can be estimated that the refolding yields for the host β-lactamase and the chimeric protein are 98% and 56%, respectively. Thermal denaturation is thus not fully reversible for BlaPChBD. As a consequence, this excludes the calculation of thermodynamic stability parameters such as ΔG.

The thermal unfolding was first investigated by differential scanning calorimetry (DSC). As suggested by the DSC thermogram (Fig. 7), the thermal transition of the chimeric protein does not fit a simple two-state model as observed by chemical denaturation. The apparent Tm and calorimetric denaturation enthalpy values deduced from these experiments are summarized in Table 4. According to the data, the hybrid protein seems to be composed of two domains characterized by distinct thermal stabilities ($Tm_{(app)}1 = 50.1° \pm 0.6°C$; $Tm_{(app)}2 = 56.5° \pm 0.1°C$) compared to the host protein, which presents only one thermal denaturation transition ($Tm_{(app)} = 59° \pm 0.1°C$). One possible interpretation of these data would identify the less stable domain with ChBD while the more stable domain would correspond to the β-lactamase. In order to verify this hypothesis, thermal denaturation of BlaPChBD was performed in the presence of DTT. Unfortunately, DTT (or TCEP) caused a strong increase of the thermogram baseline, preventing the correct determination of the denaturation parameters.

According to the deduced $Tm_{(app)}$ values obtained for the chimeric protein, the β-lactamase domain in the hybrid protein is less stable than in the original protein. The calorimetric enthalpy value (ΔH_{cal}), obtained for BlaP (511 ± 8 kJ mol^{-1}) is smaller than that of BlaPChBD (810 ± 70 kJ mol^{-1}). This can be explained by the additional heat needed to denature the additional domain of the hybrid protein.

To further demonstrate the presence of an intermediate state during the thermal unfolding process of the chimeric protein, thermal denaturation was monitored by far-UV CD and fluorescence spectroscopies (Fig. 8). For the parental β-lactamase, the data obtained by far-UV CD and fluorescence spectroscopies are virtually indistinguishable, suggesting the presence of a two-state unfolding process. The apparent Tm values deduced from far-UV CD and fluorescence spectroscopy are $58° \pm 0.1°C$ and $57.6° \pm 0.1°C$, respectively. These values are in good agreement with that determined by DSC. For the hybrid protein, only one single transition ($Tm_{(app)} = 53.1° \pm 0.1°C$) was observed by fluorescence measurements, whereas data from far-UV CD demonstrate the presence

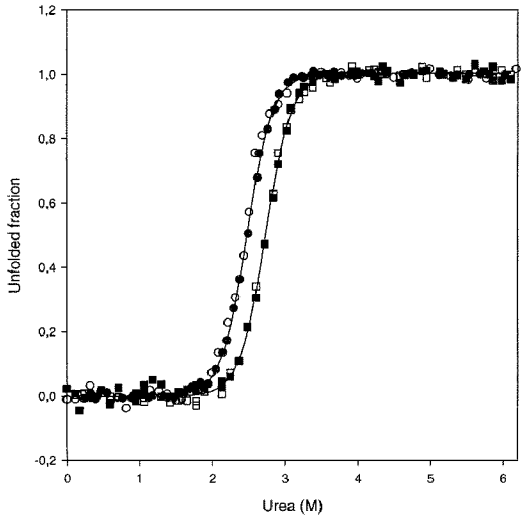


Figure 5. Unfolding transitions of the chimeric protein (circles) and the host protein (squares), monitored by intrinsic fluorescence (●, ■) and far-UV CD (○, □).

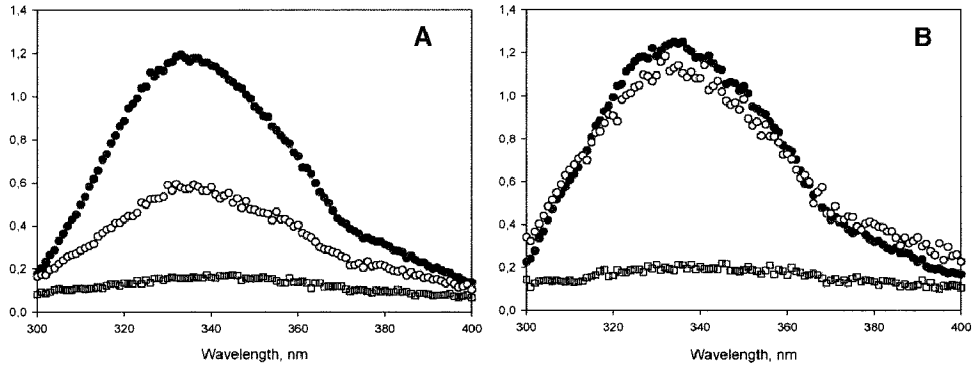


Figure 6. Intrinsic fluorescence spectra of (*A*) the chimeric protein and of (*B*) the parental β-lactamase. (●) Native state in 50 mM sodium phosphate buffer (pH 7.5); (□) unfolded state at 90°C; (○) refolded state after cooling to 25°C. The excitation wavelength was 280 nm.

of two transitions with $Tm_{(app)}$ values of 53.3° ± 0.7°C and 67.6° ± 0.3°C separated by an intermediate state. These values do not correspond to those observed by DSC ($Tm_{(app)}1$ = 50.1° ± 0.6°C; $Tm_{(app)}2$ = 56.5° ± 0.5°C). The reversibility of the first thermal unfolding transition of the chimeric protein was investigated by heating the protein to 60°C. Only 66% of the enzymatic activity was recovered after cooling the sample, indicating an incomplete reversibility of the first unfolding step.

Far-UV CD and fluorescence spectroscopy were also used to monitor the thermal denaturation of BlaPChBD in the presence of DTT. However, the reduced hybrid protein exhibited altered fluorescent properties that prevented interpretation of the transition curve. In contrast, the thermal transition could be monitored by far-UV CD (Fig. 9), showing that destabilization of ChBD by reduction of the disulfide bridges restores a single and cooperative transition for the thermal unfolding of the chimeric protein with a Tm value of 53.1° ± 0.4°C.

The enhancement of 1-anilino-8-naphthalenesulfonate (ANS) fluorescence upon binding to exposed hydrophobic regions of partially unfolded protein molecules has been used extensively to detect intermediate species in protein folding (Tsybovsky et al. 2004). Thus, in order to further characterize the intermediate state observed by far-UV CD, the thermal unfolding was monitored by ANS fluorescence. Results of ANS binding experiments to the chimeric protein and the host protein are compared in Figure 10. For the chimeric protein, the increase of temperature was accompanied by a substantial increase of ANS binding up to ~57°C. These data confirm that a partially folded intermediate exhibiting ANS-accessible hydrophobic clusters is formed upon denaturation. The much weaker increase of ANS binding observed during the thermal unfolding of the parental β-lactamase indicates a cooperative unfolding for this protein. The maximum of ANS binding is centered at ~59°C, which is very close to the unfolding midpoint.

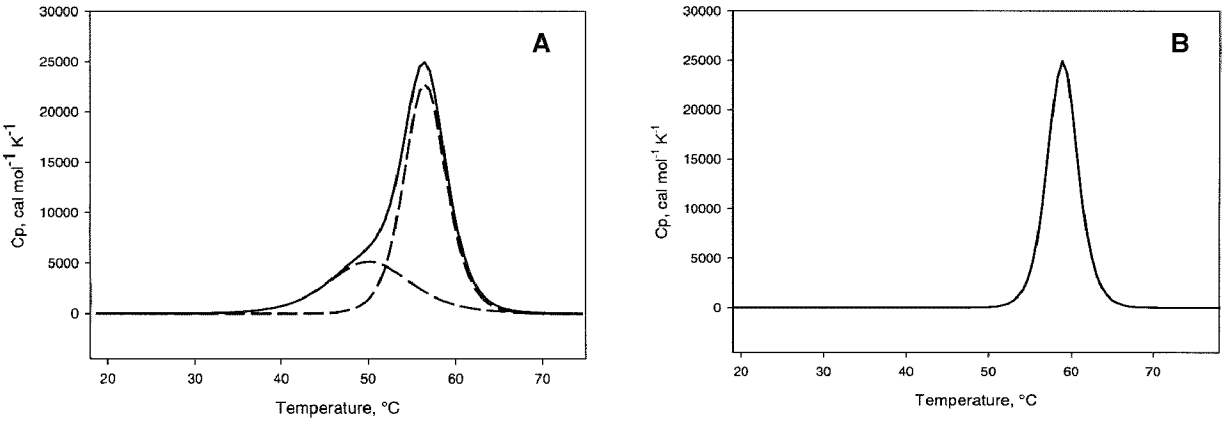


Figure 7. Differential scanning calorimetry of (*A*) BlaPChBD and (*B*) BlaP. The thermograms of the native form are presented in all panels (continuous lines), showing two domains characterized by distinct thermal stabilities (discontinuous lines) for the chimeric protein, whereas the parental β-lactamase presents a single transition. The protein concentration was 65 μM for both proteins in 30 mM MOPS buffer, 50 mM NaCl (pH 7.5).

Table 4. *Calorimetric enthalpies and apparent* Tm *values calculated for the chimeric protein and the host protein*

Protein	ΔH_{cal} (kJ mol^{-1})	$Tm_{(app)}$ (°C)
BlaPChBD		
First domain	270 ± 40	50.1 ± 0.6
Second domain	610 ± 40	56.5 ± 0.1
Total	870 ± 70	/
BlaP	511 ± 8	59 ± 0.1

Discussion

Gene fusion technology has been widely used for biological screening and purification of recombinant proteins (Ladant et al. 1992). Two kinds of connections can be used. The first and most current one is the classical "end-to-end" fusion in which the N terminus of one domain is linked to the C terminus of the other ones. The second one is "insertional" with one domain inserted in-frame into the primary structure of the parental one (Abedi et al. 1998).

The main difference between these two types of fusions is that two connections instead of one are imposed between both partners in the case of an insertion (Abedi et al. 1998). Consequently, it can be expected that two connections might render the resulting chimeric protein more rigid and stable.

However, the construction of an insertional fusion protein is rather complex, because it requires ideally structural information about both partners. In general, large insertions of structural domains can be tolerated as long as the inserted protein is introduced in a position where no steric hindrance occurs. Thus it is accepted that domains where N- and C-terminal extremities are close to each other in the three-dimensional structure are better tolerated in insertional fusion proteins (Abedi et al. 1998).

We have used this technology to insert the chitin-binding domain, ChBD, of the human macrophage chitotriosidase into a loop of BlaP, a 30-kDa β-lactamase. This resulted in a completely bifunctional chimeric protein, BlapChBD, exhibiting chitin-binding properties and enzymatic activity very similar to those of the parental proteins. ChBD was inserted into BlaP prior to any knowledge about its conformation. The conservation of the chitin-binding properties of the chimeric protein suggests a proximity of the N and C termini in the three-dimensional structure of ChBD.

Furthermore, neither the host β-lactamase nor the reduced chimeric protein can bind chitin. The chimeric protein interacts specifically with chitin, and our data confirm that the presence of the three disulfide bridges of ChBD is essential for binding properties (Betton et al. 1997; Abedi et al. 1998). The relative equilibrium association constant (K_r = 5.8 ± 0.3 L g^{-1}) between the hybrid protein and chitin is similar to those observed for other carbohydrate-binding domains. For example, the ChBD of chitinase A1 from *Bacillus circulans* WL-12 (ChiA1) (Tomme et al. 1996) and the N-terminal cellulose-binding domain of *Cellulomonas fimi* CenC1 (Hashimoto et al. 2000) are characterized by K_r values of 14.9 L g^{-1} and 5.1 L g^{-1}, respectively. It would, indeed, be interesting to compare the binding properties of the chimeric protein to those of the isolated ChBD. Unfortunately, attempts at producing the isolated ChBD were unsuccessful.

The kinetic experiments showed that the insertion of ChBD in the β-lactamase slightly modulates the accessibility, flexibility, and stability of the β-lactamase catalytic site as shown by the specific effects with different substrates. The chimeric protein retains its catalytic activity when immobilized on insoluble chitin. This result indicates that the constraints generated by ChBD

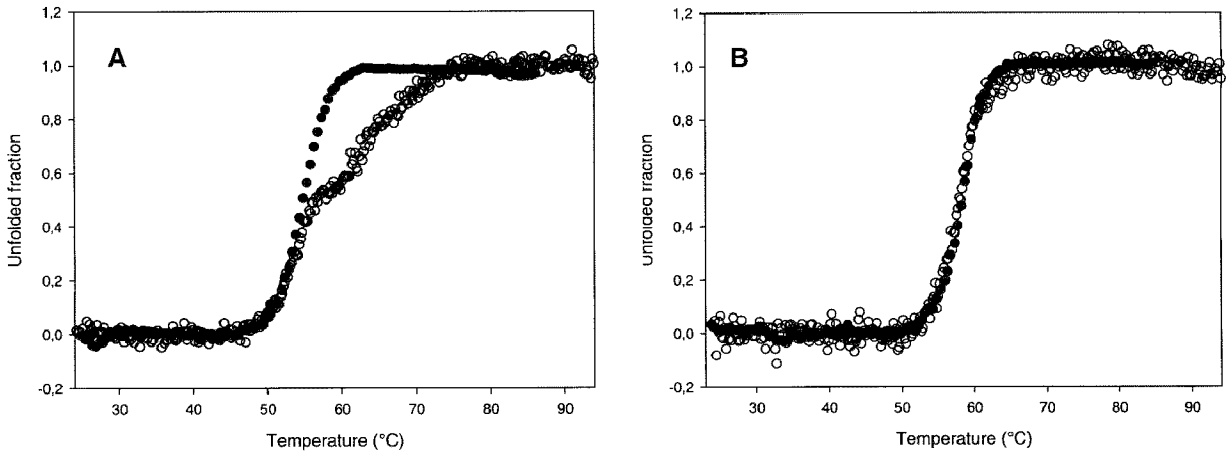


Figure 8. Thermal unfolding of (*A*) the chimeric protein and (*B*) the parental β-lactamase monitored by intrinsic fluorescence (●) and far-UV CD (○).

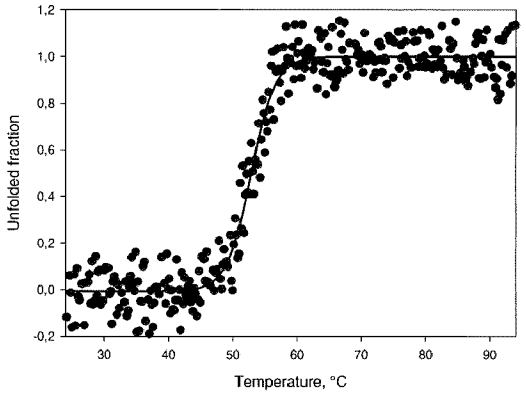


Figure 9. Thermal unfolding of the chimeric protein monitored by far-UV CD at 222 nm in the presence of DTT (100 mM).

insertion and by its binding to the target polysaccharide do not modify the flexibility and accessibility of the catalytic site. In addition, destabilization of ChBD by reduction of its disulfide bridges did not alter the β-lactamase moiety of the hybrid protein.

Stability studies

The effects of ChBD insertion on the structure and thermodynamic properties of the β-lactamase moiety were thoroughly investigated to analyze the validity of this host protein for domain insertions. Urea-induced denaturation appears to be completely reversible for both proteins so that thermodynamic parameters can be determined. The comparison of ΔG values of the chimeric and host proteins demonstrates that the insertion of ChBD slightly alters the stability of the β-lactamase, with a small decrease of ΔG (3 kJ mol^{-1}). These experiments also suggest that the insertion does not seem to modify the folding process, since both proteins exhibit a single and cooperative transition corresponding to a simple two-state model.

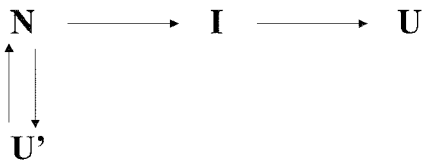
However, the analysis of thermal denaturation data yields different results. First, a refolding yield of ~50% was found after thermal denaturation of the chimeric protein, in contrast to the parental β-lactamase, which correctly refolds with a yield close to 100%. Intrinsic fluorescence spectroscopy indicates a single and cooperative transition for both proteins; however, DSC thermograms and far-UV CD clearly suggest the presence of an intermediate state during the thermal unfolding of the chimeric protein. Since neither the transition from the native state to the completely unfolded state nor the transition from the native state to the intermediate state is reversible, we suggest a general three-state model for the thermal unfolding of BlaPChBD (Scheme 1).

This suggests that ChBD insertion affects the refolding process after thermal denaturation and that a significant proportion of chimeric molecule is trapped "off-pathway" during the refolding process (Abedi et al. 1998).

The two transitions observed by far-UV CD were not detected by fluorescence spectroscopy. The difference between both methods can be explained as follows. The intrinsic fluorescence of a folded protein is a mixture of the fluorescences of its individual aromatic residues, reflecting their immediate environments. Thus identical aromatic residues do not contribute similarity to the overall fluorescence of the protein. The β-lactamase contains three tryptophans and six tyrosines and ChBD one tryptophan and two tyrosines. Thus, one hypothesis that might explain why fluorescence spectroscopy detects only one single transition upon both thermal and chemical denaturation is that only the aromatic residues in one of the partners of the chimeric protein significantly contribute to the fluorescence of the entire protein, and thus that fluorescence spectroscopy only monitors the transition of this partner. Not only does ChBD contain fewer aromatic residues than the β-lactamase, but the fluorescence of these residues might also be strongly quenched by the three disulfide bridges present in ChBD. This suggests that the β-lactamase chromophores are the major contributors to the fluorescence of the chimeric protein.

Conclusion

In conclusion, despite their vast potential, insertional fusion proteins have not been explored extensively yet, owing to the absence of standard engineering strategies (Collinet et al. 2000). Here we have successfully inserted an 8-kDa chitin-binding domain into an unrelated protein using this approach. The properties of the bifunctional chimeric protein, BlaPChBD, support the permissivity of the insertion site even when the insert contains more than 60 amino acids. The chimeric protein retains both parental activities. Our results are in good agreement with the data published by Betton et al. (1997). In addition, we also observed that the presence of an additional domain impairs a complete refolding of the hybrid protein after thermal denaturation. Nevertheless, urea denaturation of the hybrid β-lactamase was completely reversible. Thus, BlaPChBD constitutes an additional example suggesting that sequence continuity is not always required for the functional folding of a protein (Abedi et al. 1998).



Scheme 1. (N) Native state; (I) intermediate state; (U) irreversibly unfolded state and (U') reversibly unfolded state.

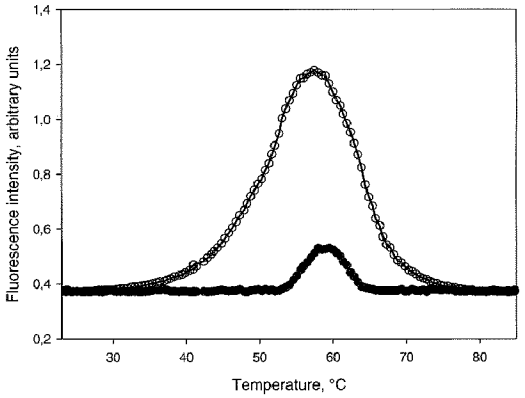


Figure 10. Thermal unfolding monitored by ANS fluorescence at 475 nm for the chimeric protein (○) and the parental β-lactamase (●). For both proteins, the concentration was 2.4 μM in 50 mM phosphate buffer (pH 7.5). The excitation wavelength was 350 nm.

These data indicate that proteins can be more tolerant to domain insertion than initially thought. As proposed by Freimuth and coworkers, this high tolerance may be a common property of the surfaces of many proteins and may have been exploited during evolution to alter molecular recognition or to modify catalytic function (Abedi et al. 1998; Freimuth et al. 1990).

Materials and Methods

Chimera construction and expression

The gene coding for the chitin-binding domain ChBD of the human macrophage chitotriosidase was constructed by recursive PCR (Prodromou and Pearl 1992) and cloned into the pGEM-T-easy vector (Promega) for sequencing. Three pairs of partially overlapping oligonucleotides were annealed separately as follows: 2 min at 95°C, 10 min at 55°C. The oligonucleotides are presented in Table 5.

Each pair of primers was mixed and annealed as described above, and 30 cycles of amplification were performed as follows: 1 min at 94°C, 1 min at 60°C, and 1 min at 68°C. Extremities of the amplified products were completed by *pfu* DNA polymerase (Fermentas) and mixed dNTP at 68°C to obtained blunt-ended DNA fragments. These fragments were inserted into the BlaP β-lactamase gene carried by the constitutive expression vector pNY-ESBlap (data not shown) in which a SmaI restriction site had been previously introduced by site-directed mutagenesis at the position selected for the insertion. The resulting genetic construct, called pNYBlaPChBD, is shown in Figure 2. The BlaP sequence is preceded by a signal peptide (pel B) for periplasmic secretion and followed by a $(His)_7$ sequence in the C-terminal position.

Expression of the recombinant fusion protein and preparation of periplasmic extracts

Transformed *Escherichia coli* K JM109 cells were incubated at 37°C in 2 L of TB medium containing 75 mg L^{-1} of spectinomycin and 10 mg L^{-1} of ampicillin. The culture was grown overnight. The periplasmic content was isolated by osmotic shock. Cells were harvested by centrifugation at 6000*g* for 10 min, suspended in 200 mL of 10 mM Tris HCl, 1 mM EDTA, and 30% sucrose (pH 8), and centrifuged at 9000*g* for 10 min. The cells were resuspended again in 50 mL of 5 mM $MgSO_4$ containing 1 mM PMSF, kept on ice during 10 min, and then centrifuged at 13,000*g* for 30 min. The supernatant was filtered and diluted twofold with 50 mM sodium phosphate buffer, 150 mM NaCl (pH 7.5; buffer A).

Purification of the recombinant fusion protein

The supernatant was applied onto a Ni-NTA-Sepharose column (Novagen; 1 × 10 cm) equilibrated with buffer A. The column was washed with seven column volumes of the same buffer, three column volumes of buffer A plus 2 M NaCl, and three column volumes of buffer A plus 10 mM Imidazole. Elution was performed by a increasing linear (10–500 mM) imidazole gradient in buffer A. Active fractions eluted at 100 mM imidazole were pooled and dialyzed against buffer B (30 mM Tris at pH 8) for the second purification step.

An anion exchange column (Mono-Q; Pharmacia; 1 × 1 cm) was equilibrated in buffer B. Elution was performed by a linear NaCl (0–1 M) gradient. The active protein eluted at 300 mM NaCl and appeared as a single band upon SDS-PAGE analysis. Protein concentration was determined using the BCA kit (Sigma-Aldrich).

N-Terminal sequencing of protein

N-Terminal sequencing was performed using the Edman degradation procedure as described by Han et al. (1977).

Mass spectrometry

Trypsin digestion followed by mass analysis of the resulting peptide mixture using matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization mass spectrometry (ESMS) of purified protein were realized in collaboration with E. DePauw's Laboratory of Mass (The Mass Spectrometry, University of Liège).

Enzymatic activity assays

The β-lactamase activity of purified proteins was measured with nitrocefin, cephalothin, and ampicillin as substrates in 50 mM sodium phosphate, 150 mM NaCl (pH 7.5) at 25°C. Specific activities and k_{cat} and K_m values of the purified enzymes were determined as described by Matagne et al. (1990).

During purification, β-lactamase activities were routinely measured with nitrocefin as substrate in 50 mM sodium phosphate, 150 mM NaCl (pH 7.5).

The Selwyn tests (Selwyn 1965) for enzyme inactivation were performed in 50 mM sodium phosphate, 150 mM NaCl (pH 7.5) at 25°C with 100 μM nitrocefin as substrate. Initial rates of nitrocefin hydrolysis were measured, with protein concentrations varying from 2 nM to 20 nM. Results were analyzed by plotting the amount of product against E_0.t, where E_0 is the enzyme concentration.

Table 5. *Nucleotide sequences of partially overlapping primers used to construct the chitin-binding domain gene by recursive PCR*

First pair:	
Sense primer (5′–3′)	
CCCTCTCCAGGACAAGACACGTTCTGCCAGGGCAAAGCTGATGGGCTCTATCCTAATCCT	
Antisense primer (5′–3′)	
ACCTGCAGCACAACTATAGAAACTGGACCGTTCACGAGGATTAGGATAGAGCCCATCAGCTTTGCCCTGGC	
Second pair:	
Sense primer (5′–3′)	
CGTGAACGGTCCAGTTTCTATAGTTGTGCTGCAGGTCGGCTGTTCCAACAAAGTTGTCCAACAGGTCTGG	
Antisense primer (5′–3′)	
AGAACGTGTCTTGTCCTGGAGAGGGGCCATGCTCAGGTTCAGAGGGCTGTCCTGG	
Third pair:	
Sense primer (5′–3′)	
CAGGGATCCATTCCAGGTACAACATTTGCAGGAGTTGCTGAACACGACCTGTTGGACAACTTTGTTGGAACAGCCG	
Antisense primer (5′–3′)	
GCCATGGGACCAGAGCTTGAAGTTCCTAAA	

Differential scanning calorimetry (DSC)

Measurements were performed using a MicroCal MCS-DSC instrument (Feller et al. 1999). Samples (≈65 μM protein) were dialyzed overnight against 30 mM MOPS, 50 mM NaCl (pH 7.5). Thermograms were analyzed using MicroCal Origin software (version 2.9), and the best fit was obtained with a three-state model in which T_m and ΔH_{cal} of individual transitions were fitted independently. In all cases, the reference cell contained the dialysis buffer. The resultant curves were corrected for baseline drift recorded when both cells contained the buffer. The scan rate was 1°C/min.

Fluorescence studies

Fluorescence spectra were recorded on an SML-AMINCO Model 8100 spectrofluorimeter (Spectronic Instruments) in a 1-cm optical pathlength quartz cuvette.

Heat-induced and urea-induced unfolding transitions were monitored by measuring the intrinsic fluorescence of the protein solution. All the experiments were performed in 50 mM sodium phosphate buffer, 150 mM NaCl (pH 7.5) with a protein concentration of 300 nM.

The fluorescence spectra were corrected for the fluorescence of buffered solutions. The excitation wavelength was 280 nm, and emission was recorded from 300 to 400 nm.

For the heat-induced unfolding experiments, the temperature was increased from 25°C to 90°C at a rate of 0.5°C/min using a programmed Lauda Ecoline RE306 water bath. When heat-induced unfolding was monitored in the presence of ANS, emission was recorded at 475 nm with excitation at 350 nm. Protein (2.4 μM) was incubated in the presence of 345 μM ANS (ε_{ANS} = 4950 M^{-1} cm^{-1}). Data were corrected for the background fluorescence of the solution in the absence of protein (buffer + ANS). The temperature was increased from 25°C to 90°C at 1°C/min, and data were collected as described above.

Urea-induced unfolding was monitored at 25°C after one night of incubation at the same temperature in 50 mM sodium phosphate buffer, 150 mM NaCl (pH 7.5). The urea concentration was varied from 0.1 to 6 M. The denaturant concentration of each sample was determined on the base of its refractive index (Pace 1986). Data were normalized using the pre- and post-transition baseline slopes as described (Betton et al. 1997). Least-squares analysis of ΔG values as a function of urea concentrations allowed the estimation of the conformational stability in the absence of denaturant, ΔG_{H_2O} ($\Delta G = \Delta G_{H_2O} - m$[Urea], with m representing a slope term that quantitates the change in ΔG per unit concentration of denaturant).

Circular dichroism

CD spectra were recorded with a Jasco J-810 spectropolarimeter, in the amide band (190–240 nm) at a protein concentration of ~4 μM in 50 mM phosphate buffer, 150 mM NaCl (pH 7.5). Spectra were acquired in a 0.1-cm pathlength cell at a scan speed of 20 nm/min, with a 2-nm bandwidth and a 4-ms response. The spectra were measured fivefold, and the baseline was corrected by subtraction of blank buffer.

Heat-induced unfolding transitions were monitored by far-UV CD at 222 nm with a Peltier holder. Experimental conditions were as above, and the temperature was increased from 25°C to 97°C at a rate of 0.5°C/min. Data were acquired every 0.2°C, with a 4-sec integration time and a 2-nm bandwidth.

Spectra of urea-unfolded samples were recorded at 25°C after incubating the samples for 16 h in 50 mM sodium phosphate buffer, 150 mM NaCl (pH 7.5). Data were recorded at a fixed wavelength (222 nm), using a 2-nm bandwidth. The values were corrected for the contribution of the solvent. The conformational stability in the absence of denaturant, ΔG_{H_2O}, was determined as above.

Binding assay

Identical amounts (25 nM) of the chimeric protein and of the parental β-lactamase were incubated during 2 h with the same amount of insoluble chitin (Sigma). The solutions were centrifuged at 13,000 rpm during 10 min to separate the supernatant containing unbound protein from the pellet containing chitin with the captured protein. The pellets were washed three times with 50 mM sodium phosphate buffer, 500 mM NaCl and then incubated in the presence of 100 μM nitrocefin for 1 min at 25°C. Then the suspension was rapidly filtered, and the absorbance of the hydrolysis product was measured at 482 nm.

K_r *determination*

Binding assays were conducted as follows: Various concentrations of the chimeric protein (25 nM to 3 μM) were incubated in the presence of 10 mg of chitin in a final volume of 500 μL of 50 mM sodium phosphate buffer, 150 mM NaCl (pH 7.5) at 22°C with continual mixing. The mixtures were centrifuged for 15 min at 4°C at 13,000 rpm, and the supernatant containing the free protein was collected. The free protein concentration was determined using the reporter β-lactamase activity. The amount of bound protein was calculated as the difference between the initial protein concentration and the free protein concentration after binding. The relative equilibrium association constant (K_r) was determined by the method described by Gilkes et al. (1992) using the following equation:

$$[B] = \frac{[N_0]K_a[F]}{1 + aK_a[F]}$$

where [B] is the concentration of bound ligand (in moles per gram of chitin), [F] the concentration of free ligand (molar), $[N_0]$ the concentration of binding site in the absence of ligand, a the number of lattice units occupied by a single ligand molecule, and K_a the equilibrium association constant (in liters per mole). The K_a value cannot be isolated from this equation, but the relative equilibrium association constant K_r (in liters per gram of chitin) is defined as

$$K_r = [N_0]K_a$$

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